Role of chondroitin sulfate proteoglycans (CSPGs) in synaptic plasticity and neurotransmission in mammalian spinal cord.

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Abstract

Chronic unilateral hemisection (HX) of the adult rat spinal cord diminishes conduction through intact fibers in the ventrolateral funiculus (VLF) contralateral to HX. Intraspinal injections of Chondroitinase-ABC, known to digest chondroitin sulfate proteoglycans (CSPGs) in the vicinity of injury, prevented this decline of axonal conduction. This was associated with improved locomotor function. We further injected three purified CSPGs into the lateral column of the uninjured cord at T10: NG2 and neurocan, which increase in the vicinity of a spinal injury, and aggrecan, which decreases. Intraspinal injection of NG2 acutely depressed axonal conduction through the injection region in a dose dependent manner. Similar injections of saline, aggrecan, or neurocan had no significant effect. These results identify a novel acute action of CSPGs on axonal conduction in spinal cord, and suggest that antagonism of proteoglycans reverses or prevents the decline of axonal conduction, in addition to stimulating axonal growth.

Key words: NG2, aggrecan, neurocan, Chondroitinase-ABC, node of Ranvier, EPSP, TTX, CNQX.

The accumulation of chondroitin sulfate proteoglycans (CSPGs) in and around the glialscar is a major obstacle for recovery after spinal cord injury (SCI) (1, 2). CSPGs consist of a core protein to which is attached one or more chondroitin sulfate glycosaminoglycan side-chains. CSPGs are involved in a variety of CNS functions, including the modulation of cell adhesion, cell migration, axonal outgrowth and synapse formation (3-7). CSPGs are found diffusely in the extracellular matrix throughout the CNS in the undamaged adult CNS, but also in a condensed matrix around neurons as perineuronal nets and in similar structures at nodes of Ranvier (8, 9). The accumulation of CSPGs in and around injury sites, particularly during the acute phase of injury, is thought to be a major barrier for axonal regeneration in the adult mammalian spinal cord and thus recovery of function after SCI (10-12). Degrading CSPGs by enzymatic removal of chondroitin sulfate chains with the the chondroitin sulphate glycosaminoglycan digensting bacterial enzyme chondroitinase-ABC (Ch'ase) promotes axonal regeneration and stimulates anatomical plasticity in the damaged and undamaged brain and spinal cord and encourages functional recovery (9, 13-17). Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation (18). Many patients with partial spinal cord injuries and preserved axons may nevertheless have no descending motor or ascending sensory function below the injury, and various factors including demyelination and inflammation have been proposed to explain the lack of conduction in surviving axons (19).

Recently we found that a lateral hemisection lesion (HX) of the adult rat spinal cord induces failure of axonal conduction through the intact axons contralateral to the HX, beginning 1 week after injury and persisting for at least 14 weeks (20). The initiation of

these physiological deficits coincided with the time that the elevated level of CSPGs in tissue surrounding the HX reached its peak (21). A similar time schedule for CSPG accumulation around the injury site has been reported in other SCI models (2, 10-12, 22). The goal of this study was to determine if the elevated level of CSPGs surrounding a spinal cord HX might be involved in the failure of axonal conduction in undamaged axons on the other side of the cord. In order to answer this question we designed two sets of experiments. Set 1 was directed towards evaluation of the ability of CSPG degradation to restore axonal conduction that normally declines contralateral to hemisection. These rats received a HX at thoracic T10 level; followed by immediate perilesional intraspinal injection of Ch'ase, or the neutral bacterial enzyme penicillinase (P'ase) as control. Following 4 weeks of behavioral testing one subgroup was used for electrophysiological evaluation of transmission through the HX level to motoneurons below the HX; the other subgroup was injected with an anatomical tracer to evaluate the survival of reticulospinal axons. Set 2 was designed to test the effects of CSPGs on axonal conduction in the intact spinal cord. Intact rats received intraspinal injections of purified CSPGs into the thoracic lateral column or around the lumbar motoneurons in uninjured cords. The acute effect of these injections on axonal conduction and on monosynaptic transmission to individual lumbar motoneurons were measured.

Our results demonstrate that the post-injury conduction block in axons close to the lesion can be prevented by Ch'ase infusion, and that NG2, one of the CSPGs upregulated after injury, can block axonal conduction and transmission to motoneurons. Some of these results have been reported in abstract form (23).

Results.

1. Conduction failure in surviving axons after chronic hemisection can be prevented by Ch'ase infusion.

1a. Acute hemisection does not affect conduction in contralateral axons.

We carried out simultaneous *in-vivo* intracellular recordings in the right L5 ventral horn of the monosynaptic EPSP in motoneurons recorded intracellularly, and field potentials recorded extracellularly, both in response to electrical stimulation of ipsilateral thoracic VLF at T7. These responses were measured before and immediately (within 10 minutes) after lateral HX of the left thoracic cord at T10 level. In uninjured rats, the peak amplitude of the maximum monosynaptic EPSP (intracellular) was 5.2 ± 0.7 mV. The extracellular response (extracellular) consisted of 2-3 peaks; an early biphasic component, representing the volley of action potentials (measured between the arrows, Fig. 1b), averaged 0.27 ±0.1 mV; the later component, representing the synaptic response, averaged 0.3 ± 0.1 mV (n = 5). After the contralateral left thoracic cord was hemisected at T10, the peak amplitude of these responses (4.6 ± 0.8 mV intracellular, 0.25 ± 0.1 mV early extracellular, 0.26 ± 0.2 mV later extracellular) were not significantly different from those measured prior to acute HX (p=0.1, n = 5, Figs. 1a, b). These results are consistent with the results of our recent study that demonstrated no significant effect of acute HX on the responses from contralateral VLF above the hemisection to motoneurons on the same side below the level of the hemisection (20).

1b. Chronic hemisection leads to conduction block in contralateral axons.

In a previous study we showed that spinal cord hemisection leads to a gradual onset of conduction block in axons contralateral to the lesion, starting at 1-2 weeks and continuing for at least 14 weeks (20). We repeated these experiments with the addition of a control infusion of penicillinase (P'ase), a bacterial enzyme with no effect in mammals, delivered to the spinal cord parenchyma on either side of the lesion at the time of operation (n=5). After 4 weeks rats were assessed electrophysiologically by *in vivo* intracellular recording from individual motoneurons and extracellular recording from the ventral horn at the lumbar L5 segment (Fig. 1). In these rats we found a marked reduction of the action potential volley and the synaptic responses evoked by stimulation of VLF above the HX level and recorded below. The mean peak amplitude of each response recorded from chronically hemisected and P'ase treated rats (Fig. 1 c, d; intracellular: EPSP peak amplitude 0.7 ± 0.3 mV; extracellular early peak: 0.04 ± 0.02 mV; later peak 0.05 ± 0.02) was significantly smaller compared to acutely hemisected rats (Fig. 1 a, b; $p =< 0.001$). Similar responses measured from chronically hemisected rats treated with P'ase were not significantly different from those recorded previously (20) in rats that received HX and no treatment for 4 weeks prior recordings ($p =0.1$, $n = 5$).

1c. Intraspinal Ch'ase attenuates conduction block adjacent to chronic spinal hemisection.

We next asked whether the decline in axonal conduction and synaptic responses that developed during the course of chronic HX (20) would still be observed if intraspinal injections of Ch'ase were administered at the time of HX. Immediately after HX, rats were injected intraspinally with either P'ase (n = 8) or Ch'ase (n = 9), and allowed to

survive for 4 weeks of behavioral assessment; they were then prepared for either electrophysiological recordings (n=5-6) or anatomical tracing (n=3). All responses measured from rats of the Ch'ase group (n = 5) were markedly larger compared to the P'ase group [intracellular: EPSP peak amplitude 2.2 ± 0.4 mV Ch'ase (vs 0.7 ± 0.3 mV P'ase), Fig. 1c, e; p=<0.001; extracellular early peak: 0.15 ±0.03 mV Ch'ase (vs 0.04 ± 0.02 mV P'ase), p=<0.001; later peak: 0.14 ± 0.04 mV Ch'ase (vs 0.05 ± 0.02 P'ase), Fig. 1d, f; p = 0.01)]. Thus Ch'ase treatment either prevents or reverses the decline in action potential conduction through VLF white matter contralateral to a chronic HX.

After completion of these recordings, rats were transcardially perfused, and 10 μm thick Cresyl Violet stained cross-sections were cut for reconstruction of the injury site, as previously described (20). We found that most injuries were uniform with a complete lesion of the left side of the cord (with the exception of one case, which was excluded from the analysis).

1d. Ch'ase has no effect on number (density) of unlesioned axons contralateral to hemisection.

To determine whether the physiological effects of Ch'ase on the conduction of axons contralateral to HX could be associated with the changes in number of reticulospinal axons, the tracer BDA was injected in the right reticular formation, and longitudinal sections were used to quantify the reticulospinal axons in the right white matter contralateral to the injury (Fig. 2 a-g). We computed the ratio of the labeled axons counted 1 cm above vs 1 cm below the HX in all consecutive sections for each rat. The

injury minimally affected these axons, and no difference in the preservation of these axons across the segment across from the injury was found between P'ase (n = 3) and Ch'ase (n = 3) groups (Fig. 2d, p=0.4).

1e. Ch'ase treatment improves locomotor behavior.

The animal's ground locomotion performance was evaluated using the open field BBB score and gait analysis. Consistent with the previously described locomotor performance of rats that received lateral HX of the thoracic cord and no treatment (20), the animals in both the P'ase and the Ch'ase groups spontaneously recover locomotor function, but the recovery process slows at about 2 weeks post-HX (i.e. the time point when the decline of axonal conduction in unlesioned axons becomes blocked) (Fig. 2). The animals treated with Ch'ase, however, showed better interlimb coordination in the Open Field (Fig. 2, h, i). Consistent with enhanced motor performance and better body stability observed using BBB, gait analyses revealed a more normal base of support of the hindlimbs in the Ch'ase group (Fig. 2j).

In summary, the results of anatomical tracing suggest that Ch'ase did not alter the survival of reticulospinal axons across the segment contralateral to chronic HX, but electrophysiological data demonstrated that the treatment partly prevented loss of conduction in these fibers. The rescue of axonal conduction, probably, accounts for the improved locomotor performance in Ch'ase treated animals.

2. Acute effects of purified CSPGs on conduction and synaptic transmission in the intact spinal cord.

The data presented above suggest that the post-lesion decline in conduction in the intact VLF could be due to an accumulation of CSPGs around the lesion site. To test directly whether increased levels of CSPGs alter axonal conduction and synaptic transmission, we injected purified CSPGs into the intact spinal cord at T10 and recorded responses in the L5 ventral horn elicited by stimulating VLF at T7. We compared responses obtained just before and immediately after the injection.

2a. In-vivo intraspinal injections of known pharmacological agents induce the predicted changes in both axonal conduction and postsynaptic potentials, recorded in-vivo from the adult rat spinal cord.

In order to evaluate if intraspinal injection is an effective delivery method with preservation of continuous long-lasting *in-vivo* electrophysiological recordings, we injected the following control substances directly into the spinal cord: saline, CNQX (an AMPA/kainate receptor blocker which selectively blocked the initial monosynaptic response of motoneurons (24), or tetrodotoxin (TTX), an inhibitor of voltage-gated Na-channels, known to diminish propagation of the action potential.

Injections of saline (1-3 µl) either in the L5 ventral horn close to the recording area, or in the thoracic T10 lateral white matter, had no effect on the evoked EPSP (intracellular) or composite responses (extracellular) (Suppl. Fig. 1, a and b). Intraspinal injections of CNQX [0.8µl(injected volume)/200µM(concentration in pipette)] close to the recording

area depressed the intracellularly recorded EPSP, about 4 minutes post-injection. In the case of the composite extracellular responses, the CNQX injections depressed only the later peak of the evoked response (denoted as synaptic component of response, see 20), while the earlier peak (denoted as action potential volley, see 20) was not affected (Suppl. Fig. 1, n = 3). At 5 minutes after injecting TTX (0.8µl /30µM) into T10 lateral white matter, all responses recorded intracellularly and extracellularly at L5 in response to stimulation at T7 were blocked (Suppl. Fig. 1, n = 5). These pharmacological experiments confirm previous suggestions (20) that the earliest biphasic component of the composite extracellular response represents the volley of action potentials, while the later component represents action potentials from neurons around the recording site activated transynaptically.

2b. Injection of NG2 in T10 white matter causes depression of EPSPs recorded from L5 motoneurons in response to stimulation of T7 VLF.

Intraspinal injections of NG2 into the T10 lateral white matter of uninjured rat (between stimulating and recording electrodes (Fig. 3f) consistent with the level of chronic HX in Ch'ase experiments (see Fig. 1) depressed the EPSP responses recorded intracellularly from lumbar motoneurons in a time- and dose- dependent manner (Fig. 3 a, e). Injections of NG2 at relatively low doses (0.8µl/0.1µg, n = 5 rats) had no significant action (Fig. 3e). Immediately after injection of NG2 at a higher dose (0.8µl/0.2µg, n = 7 rats), the amplitude of the evoked EPSPs was not changed, suggesting that the injection procedure did not damage conducting axons and the recording cells. However, 1.5-2.5 h after injection of 0.2µg NG2 into the T10 white matter, the amplitude of the EPSPs recorded

intracellularly from L5 motoneurons was significantly depressed (Fig. 3e; P=0.03). Injection of the higher doses of NG2 (0.8 µl/0.8 µg, n = 5 rats) into T10 induced a greater depression of the evoked EPSPs in L5 motoneurons, and this depression occurred more rapidly, i.e. all measured EPSP responses reached the lower steady level within about 30-45 min after the injection (Fig. 3d, e).

2c. Injection of NG2 causes axonal conduction block.

The most likely explanation for the depression of EPSPs in L5 motoneurons induced by intraspinal injections of NG2 between the recording and stimulating electrodes (Fig. 3 a, b) is failure of the action potentials to conduct past the injection site. Therefore, in addition to intracellular recordings we carried out simultaneous extracellular recordings from the L5 ventral horn and measured the effects of intraspinal injections of NG2 on the action potentials volley responses (Fig. 3 c, d). We found that NG2 injected into T10 white matter between the stimulating and recording electrodes induced conduction block, which closely paralleled the changes in the amplitude of intracellular EPSPs described above, with a similar pattern for the time and dose-dependent depression induced by NG2 (Fig. 3e).

2d. Effects of NG2 depended on the level of intraspinal injections vs position of the stimulating electrode.

To demonstrate that NG2 injections were effective only at all points between the stimulating and recording electrode but ineffective if placed outside the conduction path, we injected NG2 either into L4-L5 (near the recording electrode positioned in L5), or into

T7 (just rostral to stimulating electrode) (Fig. 4). Injections of NG2 (0.2µg, n = 5) between the stimulating and the recording electrodes, but near the recording area (L5), induced a similar degree of depression of intracellular EPSPs (48.0 ±10.8%, p=0.008) and extracellular composite responses (70.0±13.9%, p=0.04), as detected in the case of NG2 injections at T10 (Fig 4a; 4c). No effects were observed in response to injections of NG2 at T7 (Fig. 4b; 4d). These results suggest that NG2 depresses conduction at the level of the injections. This suggestion is supported by immunohistochemical results (Suppl. Fig. 2) demonstrating that NG2 injected intraspinally into T10 remains localized to the injection site. Injection of NG2 just above the stimulating electrode had no effect on the size of the intracellular EPSPs, demonstrating that NG2 does not cause conduction block by damaging axons.

2e. Injection of aggrecan or neurocan does not cause conduction block.

We next asked whether the ability to block conduction was a specific property of NG2 or a general property of CSPGs that are present at CNS injury site. We performed intraspinal injections of two other CSPGs, aggrecan and neurocan, into the spinal cord and carried out similar experiments. In contrast to the inhibitory action of NG2 injected in T10 between stimulating and recording electrodes (see Fig. 3f), similar intraspinal injections of either aggrecan (0.8 µg, p=0.3, n = 5, Fig 5 a, b, e, f) or neurocan (0.8 µg, p = 0.6, n = 4, Fig. 5 e, f) had no significant effect on the evoked responses, recorded either intracellularly or extracellularly. In rats tested initially on one side with aggrecan with no decline in conduction observed, subsequent injections of NG2 in the contralateral white matter depressed the evoked responses on that side (Fig. 5 c, d; n = 3). These results

suggest that the depression of axonal conduction is specific to NG2, but not other CSPGs studied, i.e. aggrecan or neurocan.

Discussion.

The main finding of this study is that CSPGs, either upregulated after spinal cord injury or directly injected, are able to block axonal conduction in unlesioned axons. We found that administration of Ch'ase across from the injured region at the time of HX largely prevented (or reversed) the decline of conduction that we observed after 4 weeks in untreated preparations (20) or after injections of control P'ase (Fig. 1). We also found that NG2, a proteoglycan known to be released into the zone of injury (1, 2), acutely diminished impulse conduction (Fig. 3).

Chondroitinase injected into the CNS retains some activity for around 2 weeks (25), so the treatment must have prevented the accumulation of glycanated CSPGs during the period when conduction block is first seen and this action is sufficient to maintain axon conduction even after the enzyme is no longer present.

In previous experiments, the improved neurological function shown by spinally injured animals treated with Ch'ase has been ascribed to enhanced axon regeneration and sprouting (11, 13, 15). In the present experiments Ch'ase treatment again led to improved motor function, with enhanced locomotion, although Ch'ase did not alter the number of surviving reticulospinal axons contralateral to the HX (Fig. 2). Lateral hemisection injuries have not previously been treated with Ch'ase. The present results suggest that at

least part of the functional recovery due to Ch'ase in this model may be due to improved axonal conduction which enhances transmission to cells downstream of the injury level during the chronic stage of HX injury (Fig. 1).

In order to elucidate the role of CSPGs on these changes in axonal conduction, and to discover whether these effects are general to all CSPGs or specific to one, we injected three purified CSPGs, NG2, aggrecan and neurocan into the lateral column of uninjured thoracic cords. NG2 and neurocan are upregulated after spinal cord injury (1, 2), whereas aggrecan exhibits complex changes (3, 26, 27). We found that intraspinal injection of NG2 (0.2-0.8 μg) at T10 to intact spinal cords depressed conduction and both early (action potential volley) and later (synaptic) components of evoked synaptic potentials measured in motoneurons in the L5 segment (Fig. 3). Injections made around the motoneurons at L5 caused a similar decline (Fig. 4). We also performed extracellular recordings, which showed that injection of NG2 at T10 caused a failure of action potential transmission past the injection site. Injections of NG2 rostral to the stimulating electrode had no effect on transmission, demonstrating that the conduction block is not due to diffuse axonal damage (Fig. 4). These results suggest that NG2 depresses conduction at the level of the injections. This effect therefore mimics the block of axon conduction that was observed contralateral to chronic spinal cord hemisection. In contrast, aggrecan and neurocan had no significant effect on transmission of impulses down the cord. In rats tested initially with aggrecan, subsequent contralateral injections of NG2 still depressed the evoked responses.

The depression of the action potential volley strongly suggests that the depression of synaptic responses might be a result of conduction block at the level of NG2 injection. CPSGs have not previously been implicated in axonal conduction block. How might such a block come about? Failure of conduction in large myelinated axons, such as the reticulospinal axons that form monosynaptic connections to motoneurons, must presumably indicate an event at the nodes of Ranvier, probably ultimately affecting the function of voltage-gated sodium channels. Many nodes of Ranvier in the CNS are contacted by an NG2 positive glial process and NG2 accumulates within the extracellular matrix at peripheral nodes of Ranvier (28). Moreover, two other CSPGs are found at CNS nodes, and appear to play a part in their construction (8, 29-31). The functions of NG2 as well as that of other CSPGs found at nodes of Ranvier are unknown, but it is likely that they participate in either the construction or stabilization of nodal specializations such as the partitioning of ion channels into discrete structural domains. These questions require further investigations.

Our results suggest that the decline of conduction induced by chronic hemisection is due to upregulation of the CSPG NG2 around the injury site, and that this molecule in some way affects nodes of Ranvier and their ability to conduct action potentials. These results identify a new and diverse acute action of individual CSPGs on axonal conduction, and carry potential for translational application to strengthen weak synaptic projections by manipulating CSPGs to enhance conduction of surviving axons in the injured mammalian spinal cord.

Figure Legends:

Figure 1. Representative traces of EPSPs (intracellular) and evoked potentials (extracellular) under the following conditions: a, b) after acute HX; c, d) chronic HX and P'ase treatment; e, f) chronic HX and Ch'ase treatment. Insert to show position of stimulation/recording electrodes vs HX. The extracellular responses consist of 2 components: the early biphasic arriving volley (measured between the arrows) and the later downward extracellular synaptic response (measured from baseline to peak). Note that P'ase did not reverse the effects of chronic hemisection in attenuating the contralateral volley whereas Ch'ABC did partially attenuate it (see text).

Figure 2. Effects of Ch'ase on the number of surviving reticulospinal axons and behavioral performance after chronic HX. (a-c) Longitudinal sections at the site of injury showing the complete transection of the (a) dorso-lateral, (b) lateral and (c) ventral funiculi of the left spinal cord and the preservation of the right white matter tracts. BDA was injected in the right reticular formation of the animals and the reticulospinal axons were counted in the right white matter contralateral to the injury, 1 cm above and below. (d) Summary of results demonstrating no difference in the preservation of these axons between the groups ($p= 0.4$). (e-g) Representative images of reticulospinal axons in the contralateral white matter tracts (e) rostral to HX, (f) across from HX and (g) below the HX. (h-j) The animal's ground locomotion performance 42 days after operation: (h) right hindlimb BBB score, (i) left hindlimb BBB score, and (j) base of support derived from

gait analysis. Animals treated with Ch'ase showed better interlimb coordination. (* P=0.02). Values are presented as mean ± s.e.m.

Figure 3. Effects of intraspinal NG2 injections in lateral T10 on the evoked EPSP (intracellular), and the evoked composite potentials (extracellular). a) Superimposed averaged responses: control EPSP (black), 5 min after NG2 injection (dotted red), 3 h after NG2 injections (solid red). b) Summary of results demonstrating delayed depression of evoked EPSPs after NG2. All means are peak amplitude of the responses from 7 rats, and for each rat the response was an average of maximum responses recorded intracellularly from 7 to 10 motoneurons before, 5 min after and 3 h after injections of NG2 (0.2 μg). c) Evoked composite potentials in the same rat, under the same conditions as in (a) to show depression of both action potential volley and synaptic components of evoked response by 0.2 μg NG2. d) Higher dose of NG2 (0.8 μg) induces greater depression of the action potential volley and synaptic components of evoked composite responses; averaged responses recorded before (black) and 40 min after 0.8 μg NG2 injection (dark red). e) Summary of results demonstrating dose dependent action of NG2 on EPSPs (intracellular, red columns) and action potential volley (extracellular, green columns). Ordinate represents mean ratio of the peak amplitudes of the maximum responses from 5-7 rats (7 to 10 motoneurons in each rat) before and 3 h after NG2 injections at denoted doses. f) Diagram to show the position of the stimulation/recording/injection electrodes. Symbol (*) above the brackets (b, e) represent significant difference ($p = 0.03$) between the corresponding bars. Values are presented as mean ± s.e.m.

Figure 4. Injection of NG2 (0.2 μg) close to the recording electrode induced depression of the evoked EPSPs (a, c, n = 4, p=0.008), to a similar extent as an injection of NG2 in T10. Injection of NG2 at even higher doses (0.8 μg) rostral to the stimulation electrode had no effect (b, d, n = 3). Values are presented as mean ± s.e.m.

Figure 5. Intraspinal injections of aggrecan or neurocan (both 0.8 μg; right T10) had no effect on evoked EPSPs (intracellular), and no effect on the evoked composite potentials (extracellular). a, b) Control response (black; position of recording/injection electrodes on the right side, same as described for NG2 in Fig. 3f), 5 min after aggrecan injection (dotted blue), 3 hour after aggrecan injections (solid blue). c, d) In rat initially injected with aggrecan, subsequent contralateral injections of NG2 in contralateral white matter (stimulate left T7, recorded from left L5, NG2 0.2 μg injected in left T10), depressed both the EPSP (recorded intracellularly) and composite responses (recorded extracellularly) (n = 3). e, f) Summary of results demonstrating no significant effect of aggrecan and neurocan on the evoked EPSPs (intracellular) and action potential volley (composite responses, extracellular) (see text for statistical analyses).

Supplementary Figure 1. Effects of intraspinal injections of control saline, CNQX and TTX on the responses evoked by stimulation of ipsilateral VLF and recorded intracellularly from a motoneurons (a) and extracellularly from the ventral horn (b). c) Injection diagram. Note: injections of saline (either in lateral T10 or near recording electrode in L5) had no effect; CNQX (injected near the recording electrode) blocked

EPSPs (intracellular) and selectively blocked only later component of the evoked response (extracellular); further injections of TTX (at T10) blocked all components/responses.

Supplementary Figure 2. Appearance of the NG2 injection site: a) Localization of endogenous and exogenous NG2 in the spinal cord, injection site; b) Normal appearing white matter at contralateral side injected with ACSF, same animal. Images shown are Z-stack projections of 6 images taken at 0.8μm intervals. Bar=50 μm.

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Methods.

These studies were carried out on adult (~210 g) female Sprague-Dawley rats in accordance with protocols approved by the Institutional Animal Care and Use Committee at SUNY- Stony Brook and VAMC.

Surgical procedure for the lateral hemisection and intraspinal injections of Ch-ase at thoracic level T10. Animals were deeply anesthetized with 3% isoflurane in 100% O_2. Dorsal laminectomy was performed to expose spinal cord segments T9-T11 and HX performed as previously described (20). Briefly, one tip of the iridectomy scissors was passed through the entire thickness of the spinal cord dorsal to ventral at the midline, and

the left dorsal and ventral columns were cut from lateral to the midline. To assess the effects of CSPG digestion, the rats received two intraspinal injections of either 1 µl of 100 U/ml protease-free Ch'ase (Seikagaku America, Falmouth, MA) or 1 µl of 100 U/ml of the control enzyme P'ase (Sigma, St. Louis, MO) immediately after the lesion procedure. Injections were given ipsilateral to the HX, 1 mm off the midline, 2 mm rostral (first injection) and 2 mm caudal (second injection) to the HX, using a glass micropipette (30 µm diameter, calibrated for a volume of 1 µl) attached to a 10 µl Hamilton syringe connected to a microdrive, over a period of ten minutes/injection. In each case, the tip of the injecting pipette was inserted 1 mm into the cord. The muscle and skin were closed in layers, antibiotic (Baytril, 5mg/kg, 0.1 ml sc), analgesic (Buprenorphine 5mg/kg, 0.1 ml sc), and 10 cc of sterile saline were administered subcutaneously.

Behavioral assessment. Rats were allowed to move freely and were scored during a 4 minute period for their ability to use their hindlimbs by two independent blinded observers. Joint movements, paw placement, weight support, and fore/hindlimb coordination were judged according to the 21-point BBB locomotion scale (32). BBB scoring was carried at day 4 and then at weekly intervals for 4 weeks. Additionally, the hind-paws were inked, and the animals then walked along a track lined with paper, allowing for the collection of footprints which were then used for the Gait Analysis (11). A one-way ANOVA followed by Pearson's $\chi 2$ test were used for data analyses.

In vivo intracellular and extracellular recordings in damaged spinal cord. After completing the behavioral evaluation, the rats dedicated for electrophysiological experiments were used to detect the functional connections from uncut axons passing through the region across from the HX. Rats were deeply anesthetized using a ketamine (80 mg/kg, 0.5 ml) / xylazine (10 mg/kg, 0.5 ml) mixture *i.p*. Heart rate and expired CO_2 were monitored continuously. Dorsal laminectomy of the spinal cord was performed with two openings, at thoracic T6-T8 (for placement of the stimulation electrode) and lumbar L1-L6 (for placement of recording electrodes at L5). We recorded the responses evoked by VLF stimulation rostral/contralateral to HX intracellularly from L5 motoneurons and extracellularly from the L5 ventral horn, below HX, (HX at left T10, recording from right L5 motoneurons, stimulation of right T7 VLF).

Motoneurons were impaled with sharp glass microelectrodes (3M KAcetate; 50-70 MΩ resistance) and identified by their antidromic response to stimulation of the cut L5 right ventral root. The resting membrane potential of motoneurons used for analysis ranged from -45 to -65 mV.

For extracellular recording we used tungsten electrode (impendance 300KΩ). The recording tungsten electrode was lowered into the lumbar L5 spinal cord to a depth of 1.3 mm. It was positioned to enter the cord at the dorsal root entry zone, at an angle of 20-22^{O} from vertical in the sagittal plane (tip directed rostrally).

For electrical stimulation of VLF we used a tungsten electrode, inserted in the thoracic cord on the same side as the recording electrodes. In order to determine an optimal depth for the stimulating electrode, we monitored the evoked response from the extracellular electrode while inserting the stimulation electrode. The depth of the VLF electrode from which a maximum response was evoked was usually about 0.7-0.8 mm. The intensity to evoke a maximum response usually ranged from 300-600 μA in chronically hemisected cords and 40-100 μA in the intact cords. The VLF stimulus had duration of 50 μs and was delivered at 0.1 Hz or 1Hz.

Tracing experiments to evaluate density of fibers that may form the stronger projections in ChABC-treated spinal cord. The rats dedicated for anatomical study were anesthetized as above and injected with the tracer BDA in the right reticular formation. After 14 days, transcardical perfusion was carried out and a block of T5-L2 spinal segments was used to prepare 20 μm thick longitudinal sections cut on a cryostat. Sections were processed as previously described (11, 15) and BDA-traced reticulospinal axons were counted in the right white matter contralateral to the injury. The number of labeled axons 1 cm caudal and 1 cm rostral to the segment across from the HX was expressed as a ratio, and these were compared between penicillinase and Ch'ase treated preparations using two way ANOVA for statistical comparisons.

Immunocytochemistry and confocal microscopy to evaluate distribution of NG2 after it intraspinal injections. Immunofluorescence staining of injected spinal cords with rabbit

anti-NG2 (Chemicon) was carried out as described previously (33) and images were taken on a Zeiss510 confocal microscope.

In vivo intracellular/extracellular recordings from the intact spinal cord and intraspinal injections of CSPGs. Rats were anesthetized as above and dorsal laminectomy of the spinal cord was performed with three openings, at thoracic T6-T8 (for placement of stimulation electrode in T7), T10 (for placement of injection capillary) and lumbar L1-L6 (for placement of recording electrodes in L5). Intracellular and extracellular recordings were performed as described above. After recording from 7-10 different motoneurons in each spinal cord, while maintaining the last intracellular penetration, we injected CSPG and continue to acquire intracellular EPSPs and extracellular evoked responses.

CSPGs, specifically the extracellular domain of NG2 (34), neurocan (*Millipore*) and aggrecan (*Sigma*), were injected into the lateral column of thoracic cord, between stimulation and recording sites (unless otherwise specified). For intraspinal injection we used the same method as above. After injection of CSPGs, we usually recorded for 3 hours, and after that we recorded intracellularly EPSPs from 7-10 motoneurons again. The maximum response for each cell was determined by examining responses to stimuli of increasing intensity. Peak EPSP amplitude was measured from pre-stimulus baseline to peak. Maximum responses from all motoneurons (50 consecutive responses/cell) were averaged over all motoneurons recorded in each rat before and after CSPG injections, respectively. For extracellular evoked responses, the average values of 500 consecutive

responses were measured before and after CSPG injections and compared over all animals.

We used the Axoprobe amplifier (Molecular Devices, Inc.) for intracellular recordings and a Grass P5 amplifier (Grass Instruments, Inc.) for extracellular recordings. All signals were digitized, stored on a PC and analyzed off line using PClamp 10. For statistical data analyses we used one-way ANOVA (SigmaPlot 11.0). If significant differences were observed between the groups, a Student-Newman-Keuls test was used for pairwise multiple comparisons between them.

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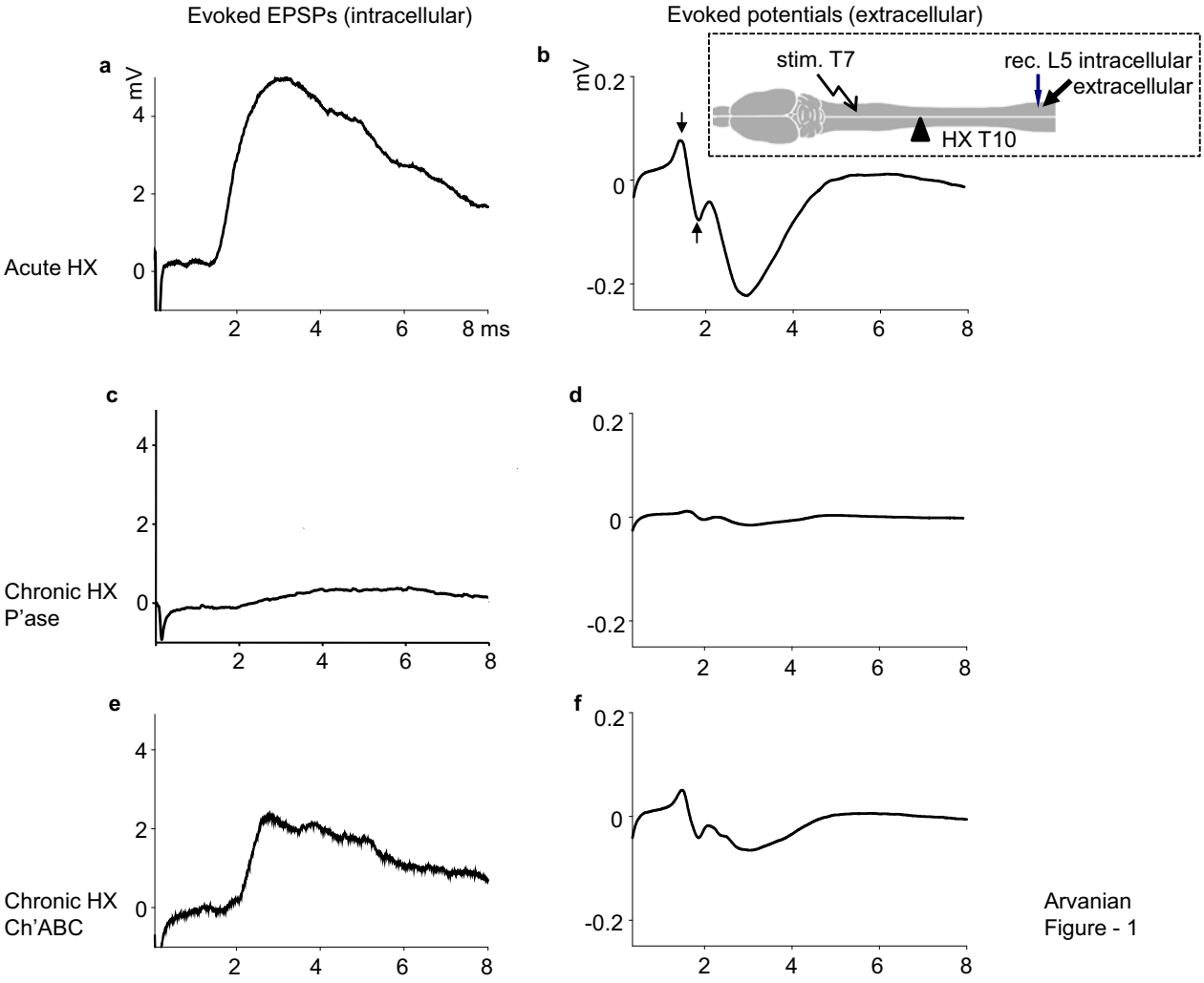

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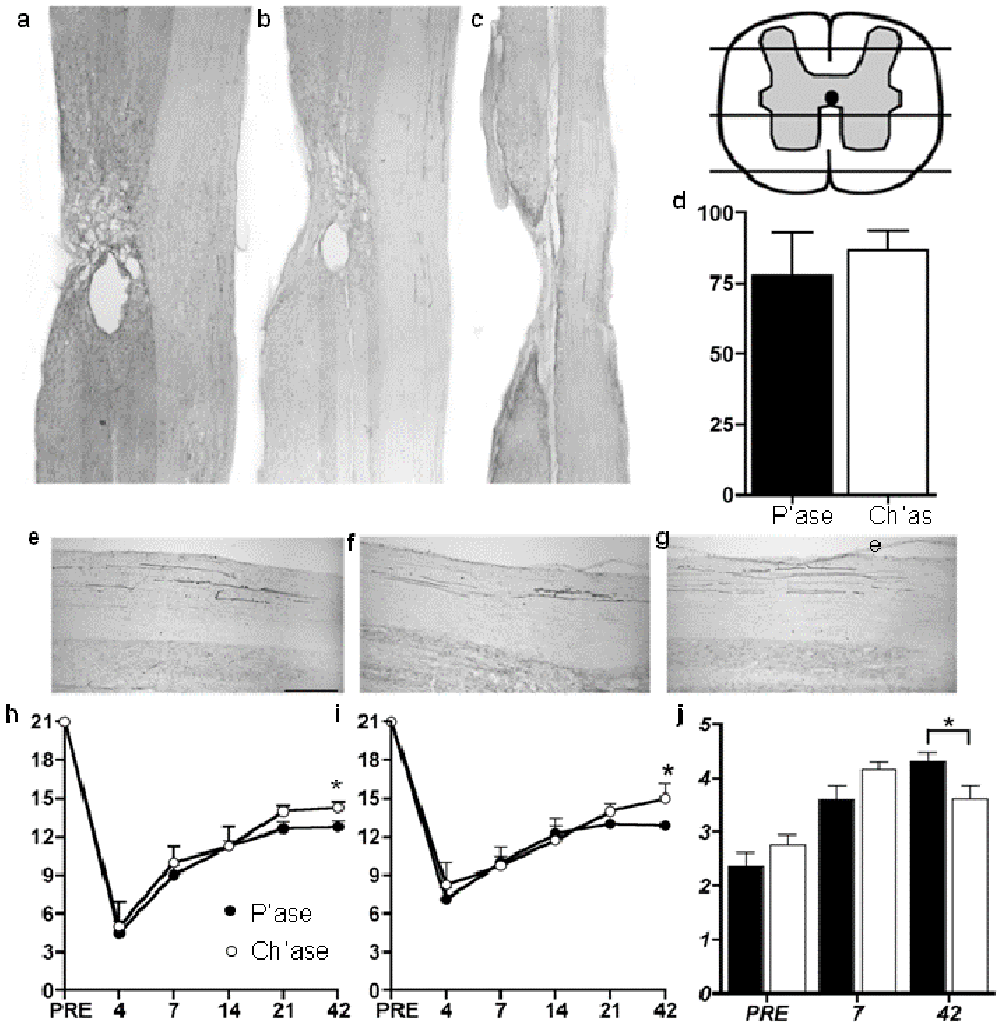

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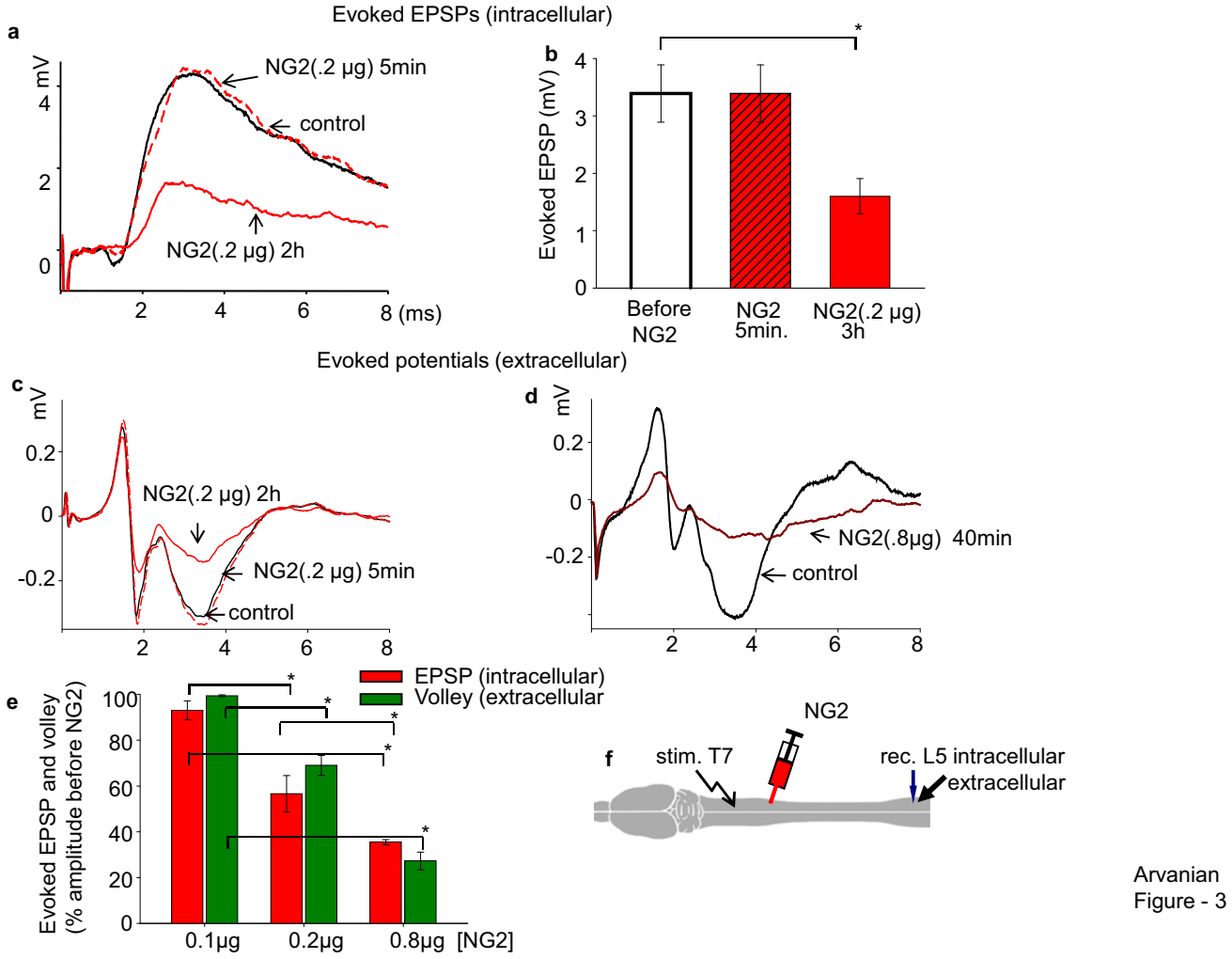

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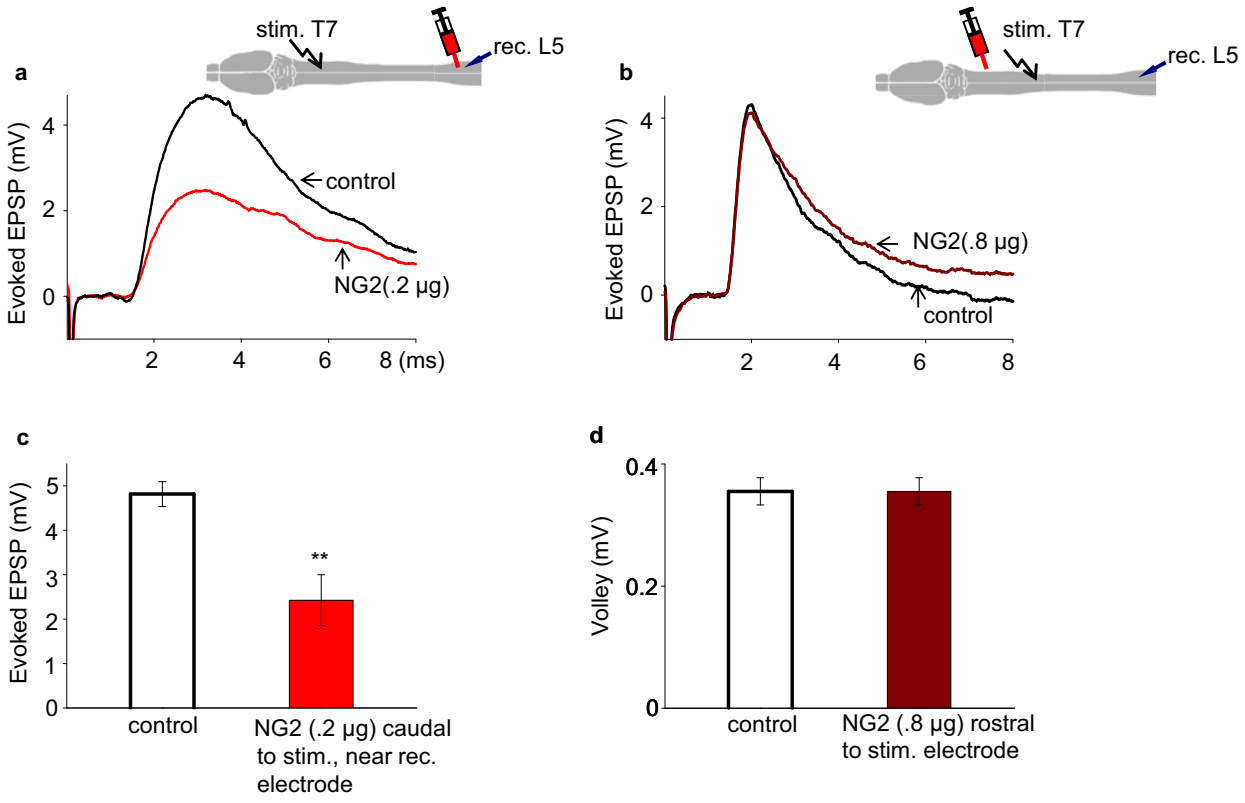

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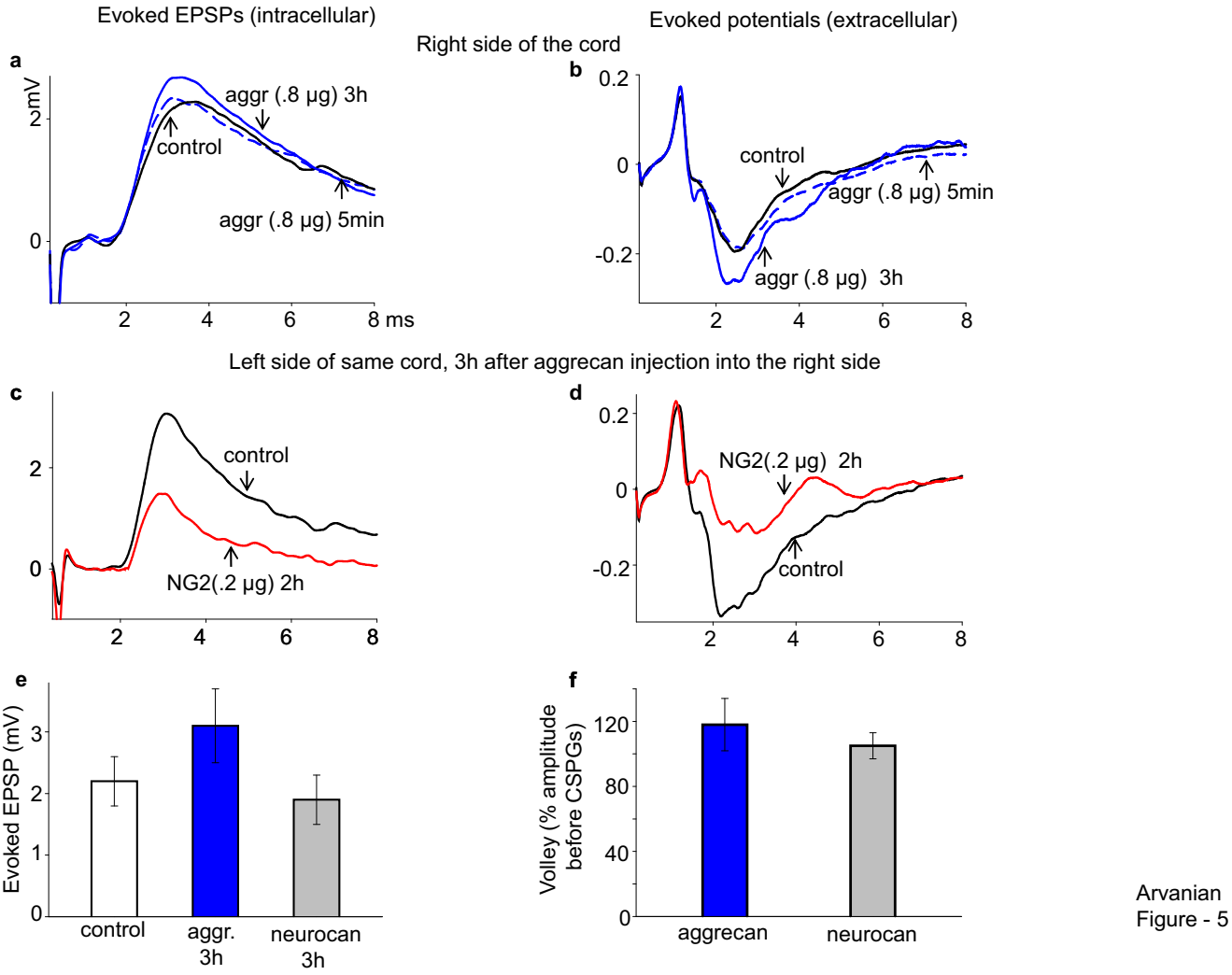

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Figure - 5